



TITLE:

Cortisol analysis of hair of captive chimpanzees (*Pan troglodytes*).

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Title: Cortisol analysis of hair of captive chimpanzees (*Pan troglodytes*)

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Abstract

In addition to behavioral evaluations, stress assessments are also important for measuring animal welfare. Assessments of long-term stress are particularly important given that prolonged stress can affect physical health and reproduction. The use of hair cortisol as a marker of long-term stress has been increasing, but there has not yet been any report on the use of such methods with chimpanzees. Therefore, the purpose of this study was to establish and validate a methodology for analyzing hair cortisol in captive chimpanzees. In the first experiment, hair was removed from the arms of nine chimpanzees living in the Kumamoto Sanctuary (KS) and the regrown hair was sampled 3 months later. Fecal samples were collected periodically during the hair-growth period. The results showed that hair cortisol level was positively correlated with the rate of receiving aggression. Although the correlation between hair and fecal cortisol levels was not significant, the individual with the highest hair cortisol concentration also had the highest fecal cortisol concentration. These results suggest that hair cortisol may reflect long-term stress in chimpanzees. In the second experiment, we investigated the physiological factors affecting hair cortisol concentrations. We cut hair from the arms, sides, and backs of 25 chimpanzees living at the KS and the Primate Research Institute. The results revealed that cortisol varied based on source body part and hair whiteness. Therefore, we recommend that hair should always be collected from the same body part and that white hair should be avoided as much as possible.

Keywords

Hair cortisol; Chimpanzees; Welfare; All-male group; Aggression

1. Introduction

Many chimpanzees are kept in captive environments around the world. A total of 325 chimpanzees were living in 51 Japanese facilities including zoos, sanctuaries, and laboratories as of 2013 [23]. In the US, 1,970 chimpanzees were living in zoos, sanctuaries, laboratories, and private houses as of 2012 [44]. A total of 1,032 chimpanzees were registered in the studbook of the European Associations of Zoos and Aquariums [12]. Chimpanzees live in a physically and socially complex environment in their wild habitats, but captive environments differ greatly from their original habitats [45]. Despite the large number of captive chimpanzees and increasing attention toward animal welfare, little is known about the link between stress and the environment of chimpanzees living in such captive environments. Understanding animal stress is important, and long-term assessments of stress are particularly important because prolonged stress often has deleterious effects on behaviors, reproduction, and physical and mental health [11, 13, 53]. However, practical constraints often prevent assessment of long-term stress in many individuals. Therefore, it is important to develop practical ways of estimating long-term stress in chimpanzees.

Cortisol is a steroid hormone released by the adrenal cortex, and its production usually increases in reaction to stressors [19]. Cortisol helps mobilize energy during stress by influencing the metabolism of sugar in the direction of increasing blood glucose levels [53]. Therefore, it is often used as a marker of stress. Cortisol assessment in chimpanzees has been performed using blood, urine, feces, and saliva [3-5, 17, 24, 30, 40, 57]. However, the use of these types of samples for estimating long-term stress presents several difficulties. For example, cortisol responds to circadian rhythms, and the peak cortisol level is found just before arousal from sleep [47]. Additionally, the

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time course of cortisol excretion in chimpanzees is short: peak cortisol levels were detected in fecal samples 22 hrs after injection of ^3H -labeled cortisol [5]. Thus, samples should be collected at multiple times. Additionally, repeated blood-sample collection can itself be stressful for animals. Recently, an increasing number of studies have validated the use of hair for stress measurement [2, 9, 15, 18, 28, 32, 49, 58]. For example, Davenport et al. (2006) found a significant correlation between hair and saliva cortisol concentrations and showed that the hair cortisol concentrations in a group of rhesus macaques increased after their relocation to another institute. Hair cortisol levels have an advantage for the assessment of long-term stress insofar as it has been hypothesized that they reflect an accumulation over a period of time. Additionally, chimpanzees often form good relationships with humans [34], which may enable us to collect hair samples relatively easily. However, there have been no reports to date on hair cortisol measurements in chimpanzees. Considering that the manner in which substances such as cortisol are incorporated into hair is not fully understood [22, 25] and that there are variations in physiology and hair features between species, it is necessary to validate the use of hair cortisol as a stress marker for each species separately and to modify the methodology appropriately.

Hence, this study aimed to investigate the validity of hair cortisol as a marker of long-term stress in chimpanzees and to examine how physiological factors such as the body part from which the hair was taken influence hair cortisol concentrations.

2. General Method

The sample consisted of 26 captive chimpanzees (16 males and 10 females) living at the Primate Research Institute, Kyoto University (PRI) ($N = 13$) and the

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Kumamoto Sanctuary, Kyoto University (KS) ($N = 13$). Basic information about each subject is presented in Table 1.

At the PRI, the subject chimpanzees lived in two mixed-sex groups in an outdoor enclosure that connected to several inside rooms. The outdoor enclosure was separated into two compartments: one was a 700-m² outdoor compound with 15-m-high climbing frames, a small stream and numerous trees; the other was a 250-m² outdoor compound with climbing frames and two small streams [35, 41]. Chimpanzees could freely access the outdoor enclosure and inside room at all times. The chimpanzees were fed seasonal fruits and vegetables, along with monkey pellets three times per day. During the experimental period, feeding-enrichment items were provided between meals on a few occasions, and cognitive experiments were conducted on some individuals [34, 36, 43].

The KS was the first chimpanzee sanctuary in Japan and is now operated by the Wildlife Research Center, Kyoto University [38]. At the KS, the subject chimpanzees lived in all-male groups. All-male grouping are not observed in wild chimpanzees, but are often formed in captivity to solve the problem of surplus animals [10, 20]. There were three all-male groups in the sanctuary, and each subject belonged to one of these groups. These individuals lived in an outdoor cage that connected to inside rooms. All outdoor cages were covered with iron mesh fences, and were about 120 m² in size and 3.8 m in height. Some grass and shrub vegetation was present on the ground. Climbing structures and several feeding devices were present in the cages for environmental enrichment. Indoor rooms were small cells 4 m² in size and 2.7 m in height. During the experimental period, the subjects were isolated in indoor rooms from evening until the next morning, but were able to communicate with neighboring individuals by visual and

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physical contact through the iron bars [38, 39]. KS chimpanzees were fed seasonal fruits and vegetables three times a day. Additionally, various enrichment efforts were made every day [29]. During the period of this study, as part of a social enrichment program, group members were changed on a daily basis. For example, one day, 15 chimpanzees were divided into two groups of 10 and five members. On another day, 15 chimpanzees were divided into three groups of five members each. Additionally, Toon and Kenny, two of the subjects in this study, arrived from a zoo in June 2008 and were initially integrated into an all-male group on June 25th, 2008. Thus, the social stimulation available to chimpanzees at KS was greater than that at the PRI, which did not offer such social variation during the period of this study.

3. Experiment 1

In Experiment 1, we examined whether the cortisol concentration in hair was correlated with that in feces and with aggressive behaviors.

3.1 Materials and Methods

3.1.1 Study site and subjects

The sample consisted of nine male chimpanzees at the KS (Table 1). Six of these chimpanzees lived in a group with daily membership changes (represented as (f) in Table 1), whereas the other three chimpanzees lived in a stable male group (represented as (s) in Table 1).

3.1.2 Sample collection

Samples were collected at the KS between May and August 2009. Hair was cut

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from the arms of the chimpanzees at the KS for the first time in May 2009 (Table 1, Fig. 1). We then waited for the growth of the new hair and collected hair from the same place in August 2009. Hair was cut by a caregiver (YM) with whom the subject chimpanzees had had extensive experience. We used scissors to reduce the stress of the sample collections. The chimpanzees voluntarily stretched out their arms and enabled us to cut near the bottom part of the hair, close to the skin surface. Fecal samples were collected periodically from individual indoor rooms in the mornings between May and July 2009. Behavioral data were obtained for 48 days between May 28th and July 31st 2009 by a researcher (NM) observing the group while group members changed daily. Sixty-minute observations were performed once per day from around 9:00–9:30 a.m. when the chimpanzees were introduced into the outdoor compound. The occurrence of aggressive behaviors including chase, charge, charging display, and attack were recorded using the focal-animal sampling method. The focal individuals were scored when initiating or receiving aggressive behaviors. The chimpanzees who were the recipients of aggression often showed responses such as scream and flee [39]. The number of aggressions was calculated as the number of bouts in which aggressive behaviors described above were seen. If an aggression ceased and started again after a while, we considered it to be two different bouts.

3.1.3 Sample preparation

3.1.3.1 Hair samples

We analyzed the hair samples collected in August. Hair samples were preserved at ambient temperature and covered in aluminum foil until assayed. The method of washing the hair and extracting cortisol from it was based on Davenport et al. [15]. Hair

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was placed in a 15-ml tube with isopropanol and shaken gently for 2 min. This washing procedure was repeated three times. After washing, samples were placed in a clean hood for approximately 5 days. The hair was ground using aPrecelly 24 (Bertin Technologies, 6500 rpm for 4 min), and 40 mg of powdered samples were weighed and placed in a 2-ml tube. One milliliter of methanol was added to each tube and incubated at ambient temperature for 24 hrs while being shaken gently with a petite shaker (Waken B Tech). After extraction, the samples were centrifuged for 15 min, and 0.6 ml of supernatant was aliquoted into a clean 5-ml tube. The extracted samples were then dried at 38 °C under nitrogen gas. The samples were reconstituted with PBS buffer before assay.

3.1.3.2 Fecal samples

After collection, 1 g of fecal sample (wet weight) was placed in a 15-ml tube with 4 ml of ethanol (80 %) and shipped from KS to the PRI. Following Khan et al. [26], the samples were not kept at ambient temperature for more than 2 weeks. As soon as the samples arrived at the PRI, the samples were stored in a biomedical freezer at –30 °C. The samples were dried at 38°C under nitrogen gas just before extraction. The extraction was performed based on the protocol for orangutan fecal cortisol extraction created by Yamazaki et al. [59]. Dried samples were weighed and thoroughly mixed with 5 ml of PBS buffer for 45 min. After being centrifuged for 15 min, 1.5 ml of supernatant was transferred to a clean glass tube. Three milliliters of di ethylether was added and then mixed for 15 min. Two milliliters of supernatant was evaporated using a vacuum dryer and then reconstituted with PBS buffer before assay.

3.1.4 Cortisol Assay

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Concentrations of fecal and hair cortisol were determined by enzyme-linked immunosorbent assay (ELISA) based on Suzuki et al. [54]. Reagents including cortisol (hydrocortisone) used as a standard, peroxidase-labeled cortisol, and O-phenylenediamine were purchased from Sigma (Tokyo, Japan). The antibody to cortisol was obtained from Cosmo Bio Co. (Tokyo, Japan). Microplates were obtained from Greiner (Tokyo, Japan). The software used for the calculation was LS-Plate manager 2000 and 2004. Any duplicates with a CV greater than 20% were removed from analysis. This resulted in some variability in the number of samples used for each individual (7–10 fecal samples per each subject). Intra- and inter-assay variability for fecal cortisol assays were 15.7 % and 28.3 %, respectively, and those for hair cortisol were 6.8 % and 14 %, respectively.

3.1.5 Statistical Analysis

Parallelism was determined by analysis of covariance (ANCOVA). When the interaction between absorbance and group (standard or sample) was not significant, we considered parallelism confirmed. For fecal samples, cortisol concentration per 1 g dry matter was calculated by dividing cortisol concentration by dry matter weight. The numbers of initiated and received aggressions per day were calculated and used for the analysis. To check the correlations between fecal and hair cortisol concentrations and between hair cortisol concentration and aggression, we used Spearman's rank correlation test. The software used for analysis was R 2.15.1 [46].

3.2 Results

3.2.1 Parallelism test

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The results of the parallelism test are shown in Figure 2. Data on the serially diluted fecal and hair cortisol concentrations were both very similar to those on the cortisol standards (Fig. 2-a, fecal samples: $F = 0.058$, $p = 0.814$; Fig. 2-b, hair samples: $F = 0.030$, $p = 0.286$).

3.2.2 Correlation between hair and fecal cortisol concentration

There was no significant correlation between overall hair and mean fecal cortisol concentration (Fig. 3-a: $r_s = 0.167$, $p = 0.678$, $n = 9$). However, the individual with the highest hair cortisol concentration also had the highest fecal cortisol concentration.

3.2.3 Correlation between behaviors and cortisol concentrations

A significant correlation between number of received aggressions and hair cortisol concentration was observed (Fig. 3-b: $r_s = 0.841$, $p = 0.036$, $n = 6$). However, no significant association between number of initiated aggressions and hair cortisol concentration was found ($r_s = -0.559$, $p = 0.249$, $n = 6$). There was no significant correlation between the rate of aggressive behavior and fecal cortisol concentration (received aggressions: $r_s = 0.319$, $p = 0.538$, $n = 6$; initiated aggressions: $r_s = -0.588$, $p = 0.219$, $n = 6$).

4. Experiment 2

To establish a practical methodology, we investigated the effects of body part, hair length, hair color, age, sex, and institutions on hair cortisol concentrations in Experiment 2. Additionally, we compared two different assay methodologies.

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4.1 Materials and Methods

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4.1.1 Study sites and subjects

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The sample consisted of 25 chimpanzees at the PRI and the KS (Table 1).

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4.1.2 Sample collection

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4.1.2.1 Hair sample collection

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From November to December 2011, hair was cut from three parts of the bodies

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(arm, side, and back) of subjects at the PRI and the KS (Fig. 1). We chose these three

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body parts because hair loss was least evident on the back and the side, and the arm was

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the easiest part from which to collect hair. The hair loss level by body part are shown in

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supplementary materials. Samples were collected in essentially the same way as they

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were in Experiment 1. It was not possible to collect samples from all three parts of all

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individuals at KS (Table 1). To check the length of hair, YY measured three randomly

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chosen hairs from each sample before washing. The average length of these three hairs

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was used as the mean length of each hair sample.

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4.1.2.2 Effects of white hair

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To check the effects of white hair on cortisol concentration, we chose six

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mixed-color samples that had been collected from the backs or sides of PRI individuals.

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We divided one sample into black and white sections and then processed and analyzed

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them separately. Additionally, YY rated the hair color of each individual by direct

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observation (see supplementary materials).

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273 4.1.2.3 Cortisol in distal and proximal sections of the hair shaft

274 To check whether cortisol concentrations in the distal and proximal sections of
275 the hair shaft differed, we randomly chose 10 hair samples collected from the arms or
276 sides of KS and PRI chimpanzees. We divided each hair into two parts by approximate
277 distance from skin surface and then processed and analyzed them separately.

279 4.1.3 Sample preparation

280 Hair sample storage and preparation methods were the same as those described
281 for Experiment 1.

283 4.1.4 Cortisol Assay

284 We used a commercially available EIA kit (expanded range, high-sensitivity
285 salivary cortisol EIA, Salimetrics LLC) and followed the manufacturer's instructions for
286 the assay. We changed the assay method because the antibody used for Experiment 1
287 was no longer commercially available, and the Salimetric kit can reduce the time needed
288 for assay. Intra- and inter-assay variability were 6.9% and 6.0%, respectively.

290 4.1.5 Statistical Analysis

291 Parallelism was determined by analysis of covariance (ANCOVA). When the
292 interaction between OD and group (standard or sample) was not significant, parallelism
293 was confirmed. We used analysis of variance (ANOVA) to test for variations in the hair
294 lengths of the three body parts. To check the effect of physiological factors and home
295 institutes on cortisol concentrations, we used arepeated-measures ANCOVA and
296 included body part, hair length, hair color, age, sex, and institute as fixed factors.To

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compare the hair cortisol concentration between white and black hair obtained from the same body region, we used the paired t test. When data were not normally distributed, we log-transformed and then Z-transformed them to achieve approximate normality. Spearman's correlation was used to check the correlations among the hair cortisol levels in the three parts of the body. The software used for analysis was R 2.15.1 [46].

4.2 Results

4.2.1 Parallelism

Data on serially diluted hair cortisol concentrations closely matched those for the cortisol standards ($F = 0.024$, $p = 0.883$).

4.2.2 Comparison of the assay methodologies used in Experiment 1 and Experiment 2

To check the correlation between the two assay systems used for Experiments 1 and 2, we analyzed cortisol concentrations in the same hair samples using the two methods. We found a significant correlation between the hair cortisol concentrations calculated using the two systems ($r = 0.98$, $p < 0.001$, $n = 8$), although the exact mean values obtained using the two systems differed: 22.7 pg/mg for the Salimetric kit and 51.5 pg/mg for the assay system used in Experiment 1.

4.2.3 Basic hair information

Mean hair length differed among the three parts of the body (arm: 5.86 ± 1.03 cm, side 5.52 ± 0.75 cm, back: 4.67 ± 1.25 cm, $F = 11.68$, $p < 0.001$). Tukey's HSD revealed that hairs taken from the arm and side were longer than were those taken from the back (arm: diff = 1.19, $p < 0.001$; side: diff = 0.85, $p < 0.01$). Hairs taken from the

back varied more in length than did hairs taken from the arm and side. Hair color information is summarized in Supp. 1-b.

4.2.4 Effects of body part, sex, age and environmental differences on hair cortisol concentration

There was a significant difference in the cortisol concentrations in hair samples taken from the three parts of the body (Fig. 4: $F = 7.83$, $P < 0.01$). Tukey's HSD revealed that the cortisol concentrations in hairs taken from the side were significantly higher than were those taken from the arm (diff = 0.593, $P < 0.01$), and the cortisol concentrations in hairs taken from the back tended to be higher than those in hairs taken from the arm (diff = 0.414, $P = 0.051$). The cortisol concentrations in hairs taken from the back and side did not differ significantly (diff = 0.179, $P = 0.557$). Correlations between the cortisol concentrations in different body parts were significant, but correlations between those in hairs taken from the back and those in hairs taken from other parts were weaker than was the correlation between those in hairs taken from the arm and those taken from the side (arm–side: $r_s = 0.708$, $n = 20$, $p < 0.001$; side–back: $r_s = 0.641$, $n = 19$, $p < 0.01$; arm–back: $r_s = 0.610$, $n = 20$, $p < 0.01$). Cortisol concentrations were higher in the KS than in the PRI chimpanzees ($F = 6.14$, $p = 0.018$). Hair length, hair color, age, and sex did not significantly influence hair cortisol (hair length: $F = 0.878$, $p = 0.355$; hair color: $F = 0.003$, $p = 0.954$; age: $F = 2.688$, $p = 0.11$; sex: $F = 0.478$, $p = 0.494$).

4.2.5 Effects of white hair on hair cortisol concentration

Comparisons of the cortisol concentrations in white and black hairs showed

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that the concentrations in white hairs were consistently higher than those in black hairs (white hair: 26.5 ± 6.91 pg/mg hair; black hair: 12.6 ± 3.40 pg/mg hair; $t = 5.39$, $p = 0.003$, $n = 6$).

4.2.6 Cortisol in distal and proximal sections of hair shaft

There were variations in the direction of the differences in the cortisol concentrations found in proximal and distal sections of hair shafts. Cortisol concentration in the distal section was higher than that in the proximal section in four of 10 samples, whereas the difference was in the reverse direction or not significant in the remaining six samples (Fig. 5).

4.2.7 Comparison of hair cortisol concentrations between 2009 and 2011

The cortisol concentration for Toon, the chimpanzee with the highest hair cortisol level, decreased in 2011 from its level in 2009 (51.1pg/mg in 2009; 21.5 pg/mg in 2011). In contrast, the cortisol concentration for George increased (8.25pg/mg in 2009; 28.6 pg/mg in 2011). Analysis of veterinary and daily chimpanzee care records revealed that Toon did not sustain any injury requiring medical treatment after 2010. Additionally, the increased cortisol concentration in George matched a period during which he refused to leave his night cage. The concentration in other individuals did not vary so much over time (average 12.7 ± 5.29 pg/mg in 2009; average 16.6 ± 3.87 pg/mg in 2011).

5. Discussion

The hair cortisol concentrations of chimpanzees can be assessed in a

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369 meaningful way. A significant correlation between hair cortisol concentration and
370 number of received aggressions was observed here. Muller and Wrangham[40] found
371 that urinary cortisol level and rate of aggressive behavior were positively correlated.
372 They also found that aggressive behavior and rank were positively correlated. This is
373 opposite to the trend found in the current study, but it is not improbable because
374 variations in the relationship between rank and stress have been reported among primate
375 species, and these variations may be related to factors such as availability of social
376 supports [1]. The subject group in the present study was relatively new, and the
377 individual with the highest hair cortisol level was one of the two individuals introduced
378 into the group a year before the study. Therefore, hair cortisol may be a good measure of
379 long-term stress in chimpanzees as well as in previously studied species. Nevertheless,
380 it was difficult to precisely determine the particular stressful period that was reflected in
381 hair cortisol levels. More detailed studies are needed to confirm the accuracy of hair
382 cortisol as a marker of stress and to precisely determine the period of stress reflected by
383 hair cortisol.

384 We did not find any significant correlation between hair and fecal cortisol
385 concentrations. There was no significant correlation between aggressive behavior and
386 fecal cortisol concentration. Although no previous reports on the relationship between
387 fecal cortisol and aggressive behavior in chimpanzees have been published, such an
388 association has been found in other animal species [e.g. 7, 14]. Additionally, changes in
389 fecal cortisol have been reported to be associated with stressful events (anesthesia,
390 relocation) in captive chimpanzees [48, 57]. Given that the collection period of fecal
391 samples matched with the period of the hair growth, sampling procedures are not likely
392 to influence cortisol concentrations. Therefore, it may have been also due to our method

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of fecal cortisol analysis that there was no significant correlation between hair and fecal cortisol concentrations. The method of fecal cortisol measurement should be refined to obtain more accurate results, given that the inter-assay variability was relatively higher than that in previous studies. Nevertheless, the individual with the highest hair cortisol concentration also had the highest fecal cortisol concentration. In addition, the differences between individuals were greater than inter-assay variability. Thus, an increased sample size may have resulted in more positive results, as in a study in dogs and cats [2]. However, considering the practical limitation on the number of fecal samples available and daily changes in cortisol level, our results might mean that hair cortisol would be a better tool to assess long-term accumulation of stress.

We cut hairs from chimpanzees rather than shaving them to reduce the stress of sample collection. Chimpanzees voluntarily stretched their arms or showed their arms, sides, or backs after being asked to do so by the person who cut their hair. In contrast with shaving, which usually requires anesthesia in non-human primates, this method allowed us to collect samples more frequently without compromising subject welfare [15, 18, 31]. Although hair cortisol has been reported to reflect long-term accumulation of cortisol in many animal species, some studies reported the possibility of short-term production of hair cortisol in hair follicles, which might indicate that sample collection can influence the hair cortisol level [25, 52]. Thus, it is important to reduce the stress of sample collection as much as possible.

We found that hair cortisol concentration varied depending on the body part from which hairs were cut. Cortisol concentrations in hairs taken from the side were higher than were those in hairs taken from the arm. However, a strong correlation was found between the cortisol concentrations of hairs taken from the side and those taken

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417 from the arm; thus, the trends characterizing changes in the cortisol levels measured
418 from these two body parts may be similar. Macbeth et al. (2010) also found
419 intra-individual variations in hair cortisol concentration in grizzly bears. They
420 considered that this variations was due to differences in timing and patterns of hair
421 growth in individuals. The cortisol concentrations in hairs taken from the side and back
422 did not differ significantly, but the strength of this correlation was weaker than that
423 between hairs taken from the side and those taken from the arm. Hair length varied
424 among individuals, and the variations between individuals were most evident on the
425 back. Thus, similarly to grizzly bears, chimpanzees might also show intra-individual
426 variations in hair cortisol concentration due to such differences in timing and patterns of
427 hair growth.

428 In addition, variations in hair color across body parts can also influence the
429 regional differences. This is because white hair was more evident on the back and
430 higher cortisol concentrations were found in white than in black hair, even when
431 collected from the same body part. Although hair color was not a significant factor in
432 the final model, it may be that the rating system used to assess black hair levels was not
433 sensitive enough to detect a relationship between hair cortisol and hair color. Supporting
434 the importance of this factor, Bennett et al. [9] also found that cortisol concentrations
435 differed in relation to coat color in dogs. They discussed the possible roles of
436 stress-associated inhibition of hair growth as well as of melanocyte development and
437 differentiation. However, it is also possible that white and black hair differ with respect
438 to the efficiency with which cortisol can be extracted given that it is well known that
439 melanin can inhibit the efficiency of DNA extraction [60]. However, because the exact
440 nature of this variation in chimpanzees was not clear, it is better to avoid white hair as

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much as possible when using hair samples as a method of cortisol measurement.

We chose these three body parts for hair cortisol analysis based on the practical convenience of collection as well as on the stable presence of hair on those sites across many individuals. Previous studies in humans and other animals have used hair from the posterior vertex region, where the lowest coefficient of variation has been found in humans [15, 22]. However, in chimpanzees, hair loss was most evident in this region, and it was not a feasible sample-collection site. Based on the results of this study, the sides or arms may provide the best hair samples for chimpanzees.

Proximal–distal comparisons revealed that individuals varied in terms of the direction of the differences between the cortisol concentrations in hairs taken from the proximal section and those taken from the distal section of the same hair samples. It is hypothesized that because cortisol accumulates during hair growth, different sections of hair differ in this regard. The results of this study support this hypothesis because the direction of difference would be more consistent if cortisol concentrations varied according to more basic factors such as absolute amount of time elapsed. Other animal studies found no significant differences in hair cortisol level between proximal and distal sections of hair [9, 15, 32]. Although some studies in humans also did not find a time-dependent change in hair cortisol level as in animal studies, other studies found a continuous decrease in hair cortisol in older hair segments [21, 27, 33]. However, in general, a significant decrease in hair cortisol concentration is most obvious beyond 6cm distal from scalp [49]. Chimpanzee hairs examined were not as long as those in humans, which might be one reason that there were no systematic change in hair cortisol according to the distance from the scalp. Sex, age, and hair length did not significantly affect cortisol concentration. However, different results may have been

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465 obtained if we had included samples from younger individuals. A previous study found
466 differences in hair cortisol concentrations among different age groups of vervet
467 monkeys [31]. Future research should analyze hair samples from more individuals
468 across a wider range of age groups.

469 We found that cortisol levels tended to be higher in the KS group, which is not
470 surprising considering the fact that these individuals lived in an all-male group, and the
471 social composition of the group changed on a daily basis. However, this does not
472 necessarily mean that the welfare of the chimpanzees at the KS was worse. Indeed,
473 all-male groups can be an alternative way of supplying social stimulation when it is
474 difficult to keep several males in a mixed-sex group. In addition, wild chimpanzee
475 societies are characterized by patterns of fission and fusion according to which group
476 companions change. For this reason, the manipulation of group membership may be a
477 good way to provide chimpanzees with a more stimulating life in a captive environment.
478 Nevertheless, effects of these husbandry procedures on stress in chimpanzees have
479 rarely been documented and it was not clear whether the challenges posed to the
480 chimpanzees by these social management schemes were at an appropriate level [11, 37,
481 42]. Toon's cortisol concentration in 2009 was much higher than that of other
482 individuals, but it decreased to a more typical level in 2011. This large change over time
483 suggests that long-term analysis of hair cortisol and comparisons among multiple
484 individuals from different social environments are necessary to understand the effects of
485 all-male group and social variability. From this perspective, hair cortisol has potentially
486 greater value than other methods that have reported stability over several years [8, 56].

487 Assessment of long-term stress is important from the view of animal welfare,
488 because long-term stress can have negative effects on welfare including behavioral

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changes, suppression of reproduction and increase of the likelihood of becoming sick [11, 13, 53]. The link between long-term stress and those welfare parameters has rarely been investigated in chimpanzees. However, there are some problems in captive chimpanzee care which can be related to long-term stress. For example, one of the leading causes of death in captive great apes is heart disease, of which the actual cause is not well understood [51, 55]. In humans, it is said that both short-term and long-term stress can increase the risk of developing heart disease [16]. Furthermore, maternal rejection and inappropriate care of infants are major problems in captive chimpanzees, and studies have shown that stress might impair maternal behaviors [6, 50]. Therefore, investigating long-term stress might shed light on the unknown causes of these important issues.

In conclusion, hair cortisol measurement is a promising tool for estimating long-term stress in chimpanzees. Hair collection should be made from the arm or side while avoiding white hair as much as possible. In combination with behavioral measures, this approach can contribute to understanding the long-term effects of husbandry procedures on the welfare of chimpanzees.

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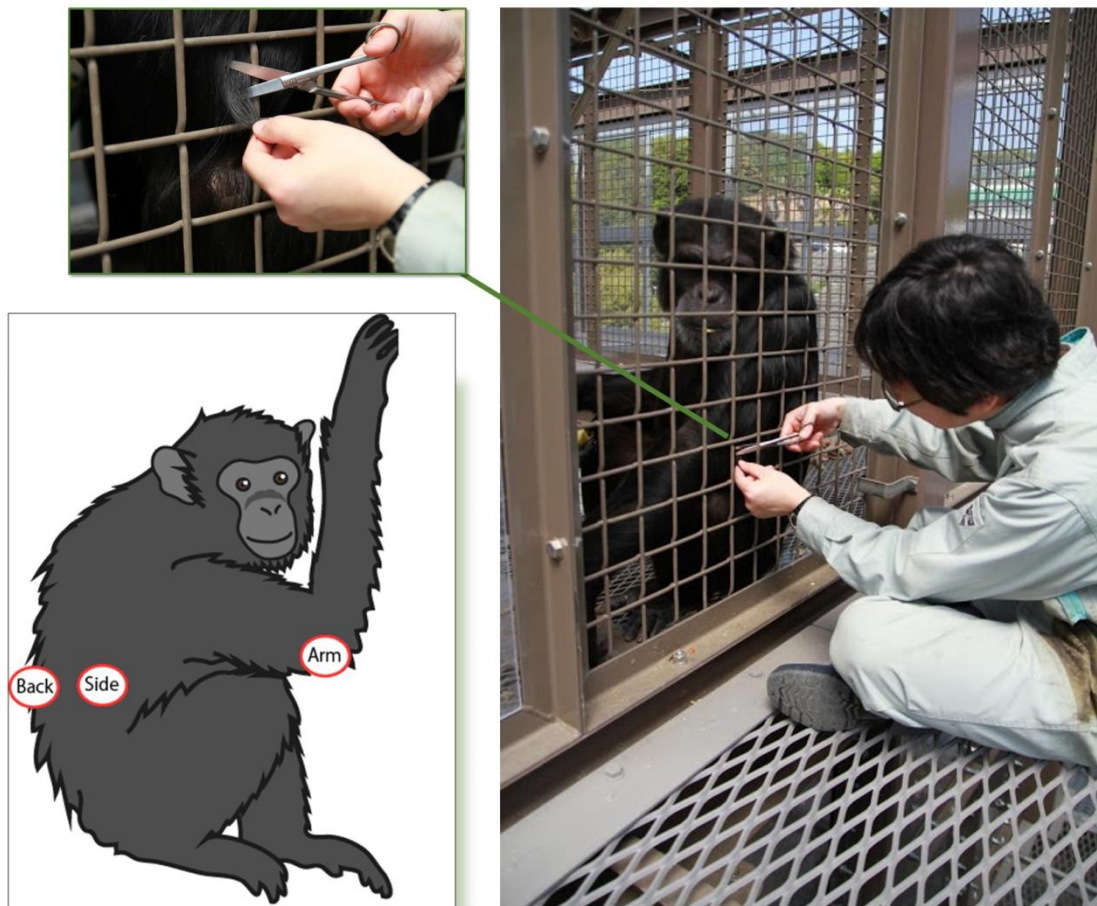
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688 **Fig 1. Illustration of sample collection and the parts of the body from which hair**
689 **samples were collected**



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Fig2. Parallelism for Experiment 1 and 2

Parallelism between cortisol standards and serially diluted samples.

Fig 2-a. The results of parallelism test of fecal cortisol and cortisol standards for Experiment 1.

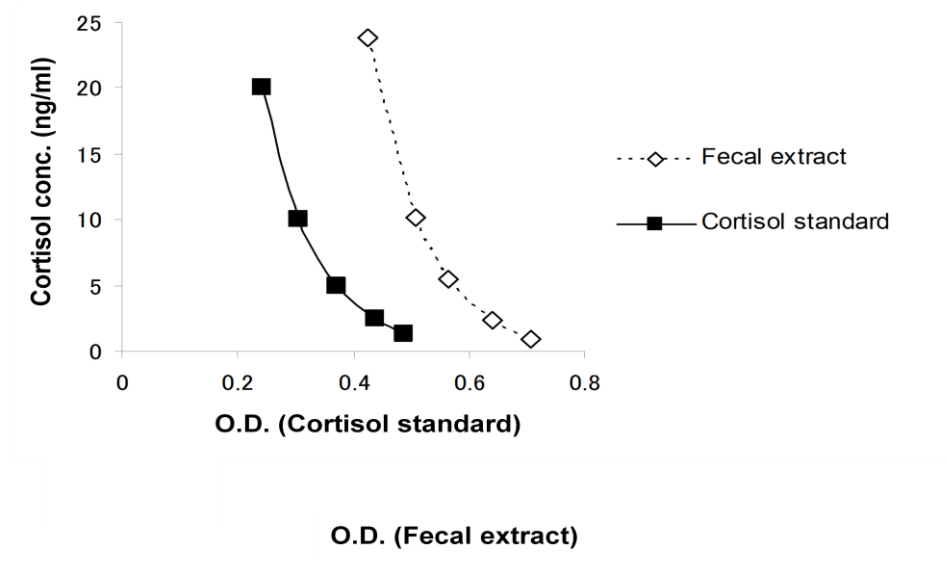
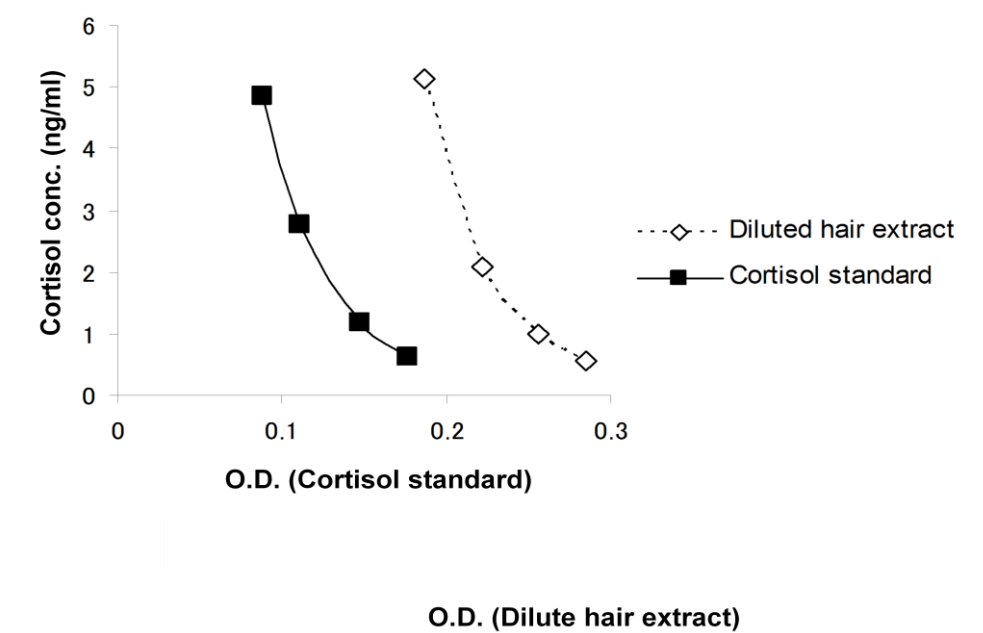


Fig 2-b. The results of parallelism test of hair cortisol and cortisol standards for Experiment 1.



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Fig 3. Correlation between hair cortisol concentration and other measures

Fig 3-a. Correlation between fecal and hair cortisol concentration

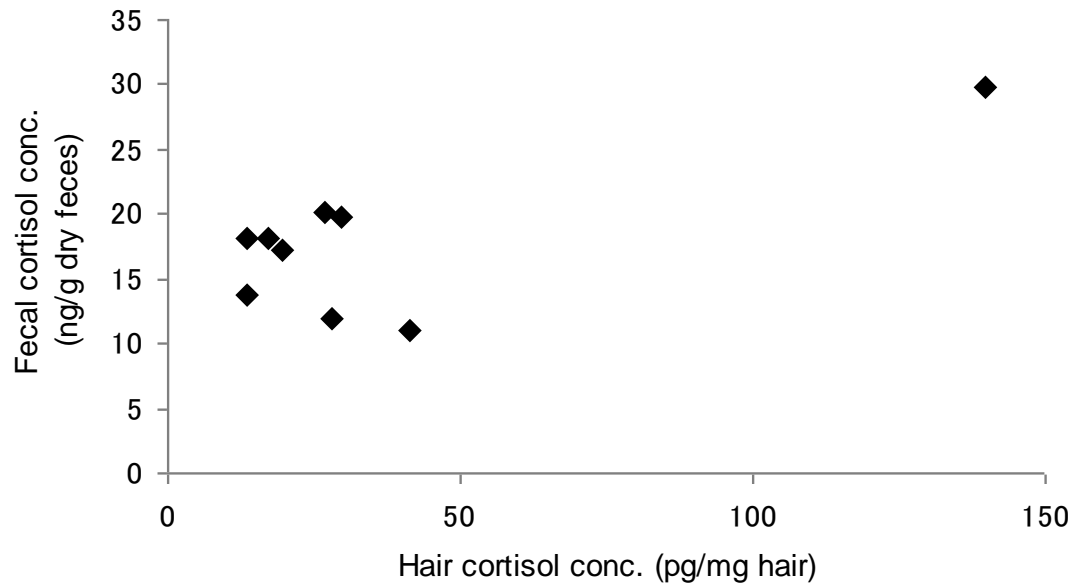
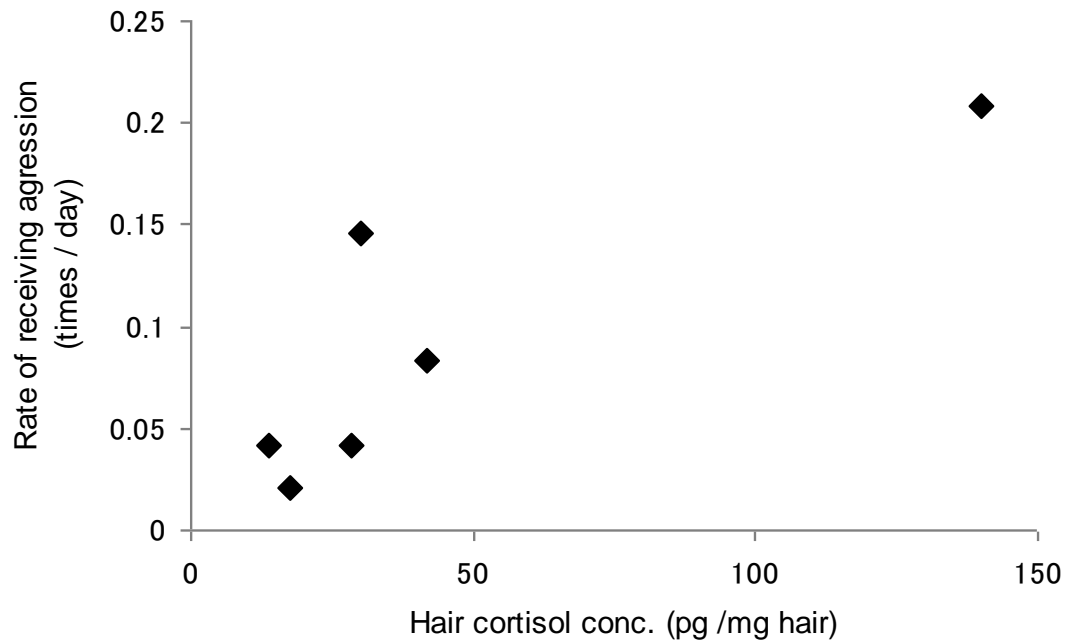
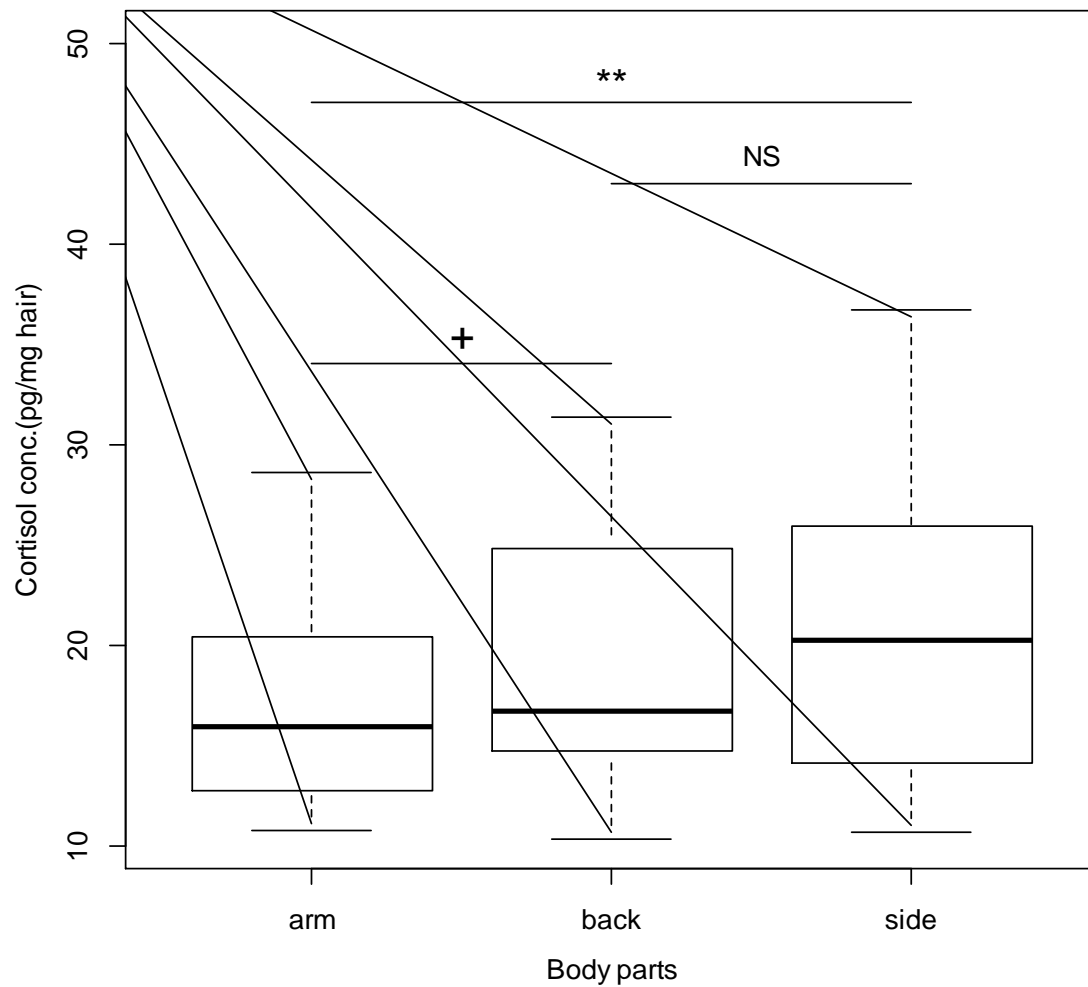


Fig 3-b. Correlation between hair cortisol concentration and the rate of receiving aggression



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708 **Fig 4. Cortisol concentration in three parts of body**

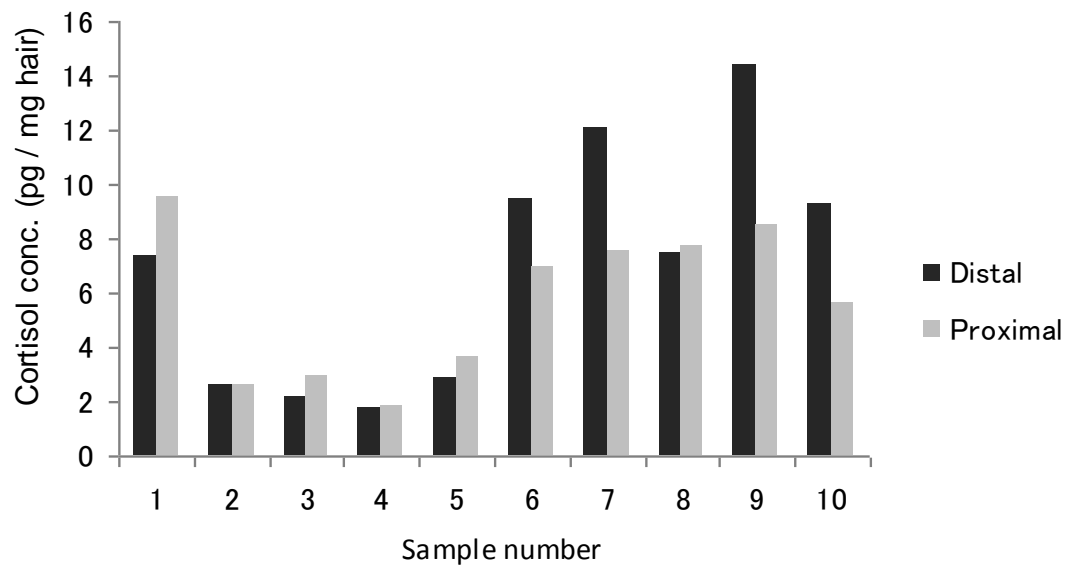


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711 **Fig 5. Proximal-distal comparison of cortisol concentration**



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